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Staphylococcus Efflux *msr*(A) Gene Characterized in *Streptococcus*, *Enterococcus*, *Corynebacterium*, and *Pseudomonas* Isolates†

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The staphylococcal *msr*(A) gene, coding for a macrolide efflux protein, was identified in three new gram-positive genera and one gram-negative genus. These *msr*(A) genes shared 99 to 100% identity with each other and the staphylococcal gene. This study demonstrates that the *msr*(A) gene has a wider host range than previously reported.

Macrolide resistance is typically due to the acquisition of genes coding for rRNA methylases, macrolide efflux proteins, and/or inactivation enzymes (10, 12, 15) (http://faculty.washington.edu/marilynr/). The use of macrolide-lincosamide-streptogramin (MLS) antibiotics has increased over the last 20 years and has been correlated with an increase in bacterial resistance to macrolides, due primarily to acquisition of a new gene(s), usually on mobile elements (12, 15). Sixty-four different acquired MLS resistance genes have been identified, with 14 of these genes found in more than two genera and 50 identified in one or two genera. The *msr*(A) gene codes for an ATP transporter that transports erythromycin and streptogramin B from the cell using energy from ATP hydrolysis and has been identified only in *Staphylococcus* spp. (9, 12–15).

Recently another gene, *msr*(D), with similarities to the *msr*(A) gene has been found downstream of another macrolide efflux gene, *mef*(A), and both genes have a wide host range encompassing both gram-negative and gram-positive genera (1, 7, 10) (http://faculty.washington.edu/marilynr/). Therefore, we hypothesized that the *msr*(A) gene may also have a wider host range. To test this hypothesis, 1,125 *Streptococcus* spp., 226 *Staphylococcus* spp., 193 *Enterococcus* spp., and 100 gram-negative isolates from oral samples, as well as 566 *Staphylococcus* spp., 160 *Enterococcus* spp., and 100 gram-negative isolates from urine samples (3, 7, 8), were screened using DNA-DNA hybridization (4, 7). The isolates were randomly selected commensal bacteria collected between 1997 and 2004 from healthy children in Lisbon, Portugal, in a randomized study aimed at assessing the safety of low-level mercury exposure from dental amalgam restorations (3, 7, 8). The isolates were previously identified using biochemical methods (3, 5). We also included 17 macrolide-resistant (Em^r) *Corynebacterium* spp. isolated from skin cultures in 1997 from patients attending an acne clinic at the University of Leeds, United Kingdom (2).

Forty-two *Staphylococcus* spp., 2 *Staphylococcus aureus* spp., 5 *Enterococcus* spp., 10 *Streptococcus* spp., 7 *Pseudomonas* spp., and 1 *Corynebacterium* sp. were positive for the *msr*(A) gene. All the positive *Enterococcus* spp., *Streptococcus* spp., *Corynebacterium* sp., *Pseudomonas* spp., and a selected number of the *Staphylococcus* spp. were used as templates in an *msr*(A) PCR assay with *S. aureus* RN4220 carrying a cloned *msr*(A) gene as the positive control (Table 1) (13, 14). All of the isolates tested produced PCR products of the correct size which hybridized with an internal probe. Thus, the *msr*(A)-positive staphylococci represented 5.6% of the isolates, the one *msr*(A)-positive *Corynebacterium* sp. represented 5.9% of the small number of Em^r *Corynebacterium* spp., and the *msr*(A)-positive *Enterococcus* spp. and *Streptococcus* spp. represented 1.7% and 0.9% of the isolates, respectively. We also identified 7 (10%) *msr*(A)-positive *Pseudomonas* spp., including 2 *Pseudomonas aeruginosa* spp., out of 69 urine isolates examined. A similar number of oral *Pseudomonas* spp. from the same population were also screened, but none were positive for the *msr*(A) gene.

We had previously examined these isolates for other MLS genes (2, 3, 7) and found that 57% of the *Staphylococcus* spp. carried at least one *erm* gene, which codes for an rRNA methylase enzyme that confers resistance to macrolides, lincosamides, and streptogramin B (2, 3, 12, 15), and that all of the other *msr*(A)-positive isolates carried at least one *erm* gene and/or *mef*(A) genes. The seven *Pseudomonas* spp. carried from one to five other MLS genes (Table 2).

To determine whether these genes were closely related to the *Staphylococcus msr*(A) genes, one *msr*(A)-positive isolate from each of the four genera, *Corynebacterium*, *Enterococcus*, *Streptococcus*, and *Pseudomonas*, was selected, and the complete structural *msr*(A) gene was sequenced using PCR assays and primers indicated in Table 1. The PCR products were confirmed using an internal ^{32}P-labeled probe, cloned into the pCRT7/NT-TOPO vector (Invitrogen, Carlsbad, CA), and transformed into *Escherichia coli* TOP10 following the manufacturer's instructions. All sequencing was carried out at the University of Washington, Department of Biochemistry Sequencing Facility, as previously described (7, 8). The *msr*(A) genes from the *Corynebacterium* sp. (AY591760), *Enterococcus* sp. (DQ068449), and *Streptococcus* sp. (DQ131177) were indistinguishable at the DNA and amino acid levels from each other and the *Staphylococcus msr*(A) gene (GenBank accession no. AB016613). The *Pseudomonas msr*(A) gene (DQ068450) shared 99% identity at the DNA and amino acid

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† Supplemental material for this article may be found at http://aac.asm.org/.

TABLE 1. Primers used for various PCR assays and expected size of the PCR product[a]

Region(s)	PCR assay primer	Sequence (5′ to 3′)	Size (bp)
msr(A)	*msr*A-Int	GCG CTC GTA GGT GCA AAT GGT	563
	*msr*A-R_2	GAT CGG TTA TGG TAC TAT TGT TA	
Internal primer	*msr*A-Int 2	GAA GAC ATG CGT GAC GTT TC	
Upstream	*msr*A-F_3	GAT CTT TGT ACT TAG AGA TAT	887
	*msr*AF-REV	TTA AAC ACT CTT ATT GTG CC	
Upstream and *msr*(A)	*msr*A-F_3	GAT CTT TGT ACT TAG AGA TAT	1,344
	*msr*A-Int REV	ACC ATT TGC ACC TAC GAG CGC	
Internal primer	*msr*A-F_2	GAC AGA TTT ACG ATC ACT TAA CAT	
msr(A)	*msr*A-F	GGC ACA ATA AGA GTG TTT AAA GG	939
	*msr*A-R	AAG TTA TAT CAT GAA TAG ATT GTC CTG TT	
Internal primer	*msr*A-Int	GCG CTC GTA GGT GCA AAT GGT	

[a] All PCR assays used 2 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), 200 μM (each) deoxynucleotide triphosphates, 1× PCR buffer (1.5 mM $MgCl_2$), 100 ng of each primer, *msr*A-Int and *msr*A-R_2, and 200 to 400 ng of whole DNA as the template. The PCR assay had an initial denaturation at 96°C for 3 min, followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 40°C for 1 min, and extension at 72°C for 2 min, with a final extension step of 72°C for 10 min. Internal primers were used for DNA-DNA hybridization with the PCR products to verify that the sequences expected were present. All primers are from the current study.

level with this *Staphylococcus msr*(A) gene and had two amino acid changes at positions 59 and 430 (see Fig. S1 in the supplemental material). The variability found in the *Pseudomonas msr*(A) gene is within the range (98 to 100%) found among different *Staphylococcus msr*(A) genes (http://faculty.washington.edu/marilynr/).

The upstream region of the *msr*(A) genes in staphylococci is thought to be involved with regulation. Therefore, it was of interest to determine whether this upstream region was also present upstream of the *Pseudomonas msr*(A) structural gene. A PCR assay, consisting of the primer *msr*A-F_3 located 328 bp upstream of the ribosome binding site and the primer *msr*AF-REV located 544 bp downstream from the start codon of the *msr*(A) structural gene, was used to generate the sequences upstream of the start codon of the *Pseudomonas msr*(A) gene, which were indistinguishable from the upstream sequences of the *Staphylococcus msr*(A) gene (see Fig. S2 in the supplemental material). The upstream and complete *msr*(A) gene sequences are at GenBank, accession no. DQ068450.

TABLE 2. Identifications of the *msr*(A) gene by DNA-DNA hybridization

Organism(s)	No. of isolates with *msr*(A) gene			No. of isolates with other MLS gene(s) (%)	Other MLS gene(s) present[a]
	Skin	Oral	Urine		
Gram-positive					
Corynebacterium sp.	1	0	0	1	*erm*
Enterococcus spp.	0	5	1	6	*erm*, *mef*(A)
Streptococcus spp.	0	10	0	10	*erm*, *mef*(A)
Staphylococcus spp.	0	8	34	24	*erm*
S. aureus	0	2	0	1	*erm*
Gram-negative					
Pseudomonas spp.	0	0	7	7	*erm*, *ere*(A), *ere*(B), *mph*(A), *mph*(B), *mph*(C)
All	1	25	42	49 (72)	

[a] Multiple different *erm* genes were found. The *erm* and *mef*(A) genes were previously identified in the gram-positive isolates (2, 3) while the *ere*(A), *ere*(B), *mph*(A), *mph*(B), and *mph*(C) genes were previously identified in a variety of gram-negative isolates (7).

A second *Pseudomonas* sp. *msr*(A) gene has now been partially sequenced and has the same high level of homology with the *Staphylococcus msr*(A) gene as the first gene sequence. We have also recently verified the presence of an *msr*(A) gene in an *Enterobacter* isolate from this same collection, suggesting that other gram-negative genera may also carry this gene. The *Enterobacter* isolate has an erythromycin MIC of 128 μg/ml using standard CLSI (formerly NCCLS) methods (6) and does not carry any of the other known gram-negative MLS resistance genes (7).

There have been 64 different acquired MLS genes identified in bacteria; however, only 17 (27%) of these genes are found in more than a single genus (http://faculty.washington.edu/marilynr/). As illustrated in this and other studies (3, 7, 10, 11), if MLS genes are screened in new genera, they are often found. Thus, screening for the presence of the *msr*(A) gene should be considered when examining the distributions of MLS genes in either gram-positive or gram-negative isolates.

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